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TITLE: The Failure of Repair Enzymes in the Catechol Estrogen-Induced DNA Damage as Potential Initiating Event

PRINCIPAL INVESTIGATOR: Ms. Kimberly A. Chapman

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

REPORT DATE: June 1999

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PREPARED FOR:
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13. ABSTRACT (Maximum 200 words) <p>This report focuses on the ability of catechol estrogen-quinones to act as endogenous carcinogens, in that, they can cause DNA damage. Studies using Matrix-assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) mass spectrometry indicated that estrogen-2,3-quinone can form stable adducts in 18 base oligonucleotides containing a single reactive site. Similar studies were carried out to demonstrate apurinic site formation in the same type of 18 base oligonucleotides after estrogen-3,4-quinone treatment. Since failure to repair or mis-repair is necessary for DNA damage to form a mutation, studies using apurinic site containing oligos were used to examine <i>in vitro</i> repair of apurinic sites. In addition, <i>in vivo</i> repair assays using apurinic-site containing pEGFP plasmids are, currently, being carried out.</p>				
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Introduction:

These studies are designed to investigate the role estrogens play in DNA damage, and how this damage affects repair mechanisms. Endogenous estrogen can be activated by endogenous enzymes (i.e. cytochrome P450s and peroxidases) to activated forms, including catechols and quinones, which react with DNA resulting in DNA damage (1-3). How repair mechanisms approach this damage determines whether a mutation is set, passed on to subsequent daughter cells, or avoided (4). Mis-repair or failure to repair certain DNA damage sequence motifs or DNA damage combinations can lead to mutations in critical genes and subsequent development of cancer (4). Estrogen levels are greatly increased in female reproductive organs, and so, the risk of cancer development in these organs is theoretically greater than in non-estrogen-dependent organs, making this a useful study in terms of breast cancer.

Annual Summary Body:

Technical Objective #1: Unscheduled DNA Synthesis in ACI rat mammary gland. This objective was completed and reported in the last report.

Task 1: completed

Task 2: completed

Technical Objective #2: Determination of up-regulated repair enzymes in human cell culture.

Task 3: Establish appropriate cultures of human breast cells that correspond to cell type determined in task 1. **Completed** (MCF-10 1A, T47D and MDA-MB-468).

Design and obtain oligonucleotides to be used as probes for enzymes that are recognized to play a role in repair, i.e. human AP endonuclease, dRAse, 5'→3' exonuclease.

Completed

Task 4: Probe for expected enzymes and determine when they appear during the cell cycle. **Not Started.**

It was decided to postpone task 4 (probe for expected enzymes to determine when they appear during the cell cycle) of this technical objective until useful DNA vectors containing DNA damage were developed. Confirmation of DNA damage by metabolites of catechol estrogens in the oligos was necessary prior to attempting task 4 in technical objective #2, as well as the entire Technical Objective #3. This confirmation procedure is discussed under the heading Technical Objective #4. In addition, Technical Objective #4 also describes the oligos and plasmids that will be used to assay for up-regulated repair enzymes (incorporated in Task 3 of this technical objective).

Technical Objective #3: Determination of up-regulated repair enzymes in human breast from women with and without breast cancer.

Task 5. Not Started.

This technical objective cannot be started until Technical Objective #2 is completed or at least begun.

Technical Objective #4: Determination of effects of different catechol estrogen-induced damages on repair enzyme function.

Task 6: Design and obtain oligonucleotides (18mers). **Completed**

Design GFP plasmid template and make. **In process; some portions have been completed.**

Establish MCF-10 1A culture for repair assay and set up *in vitro* repair assay. **Completed.**

Task 7: Determine best conditions for repair assay and carry out assay with all the designed oligonucleotides. **In Process**

Learn microinjection of MCF-10 1A cells and determine best conditions. **In process.**

Task 8 Carry out DNase foot printing to determine location of repair subunits in relation to DNA damages. **Beginning**

To determine the effects of catechol estrogen-induced damage, an effective method to determine levels of damage was needed. The first attempt focused on using an apurinic site assay previously described in the literature (5). This procedure could not be made to work. A second approach uses gel electrophoresis and has been marginally successful. Finally, a mass spectrometry technique was developed and is very useful to demonstrate DNA damage directly. In addition, gel electrophoresis approaches have been used to identify *in vitro* repair catalyzed by cell extracts and an *in vivo* approach is being designed.

Many of the studies in which identification and quantitation of damage were undertaken used 18 base long oligonucleotides (oligos), which were designed and described in the previous report. They are reiterated in Table 1. These oligos were designed with several issues in mind. Primarily, they are 18 bases long which is long enough for the catechol estrogen to react and for the initial binding of AP endonuclease or ERCC1, XPD, and XPB, required for repair of these oligos. Double stranded oligos can be created by mixing complementary strands in a complementation buffer (50mM Tris-HCl, pH 7.5, 50mM NaCl, 5mM EDTA), heating them to 90°C, cooling them quickly in ice, bringing the NaCl concentration to 150mM, heating at 50°C for 30 min., and then, allowing the oligos to anneal overnight.

Table 1: 18 base oligos used for MALDI-TOF analysis

Oligo name	Sequence	Parent <i>m/z</i>
CT9A	5'-CTTTCCTCATCTTCCCTT-3'	5301
CT9G	5'-CTTTCCTCGTCTTCCCTT-3'	5317
GA10dU	5'-AAGGGAAGAdUGAGGAAAG-3'	5700
GA10C	5'-AAGGGAAGACGAGGAAAG-3'	5677

Oligo GA10dU was designed to contain a deoxyuridine in the 10th position of an 18mer, so that the enzyme Uracil DNA Glycosylase (UDG) could be used to create an apurinic site. This oligo acts as the positive control for all the protocols that utilize these oligos. An oligo containing dU treated with UDG is also utilized in some of the cell extract repair studies to guarantee the

formation of an apurinic site at a particular location.

Two other oligos, CT9A and CT9G, are designed to contain a single purine base with which a catechol estrogen-quinone may react. In the following studies, estradiol-2,3-quinone (E-2,3-Q, 21.8mM in acetonitrile) or estradiol-3,4-quinone (E-3,4-Q, 21.8mM in acetonitrile) were reacted with either oligo (20ug) for 5 min to 16 h.

Initial studies were undertaken using the apurinic site assay described by Kubo, et al. (5-7). This approach utilizes the chemical substrate, N-epsilon (+)-biotinyl-L-lysine hydrazide (ARP, Fluka and Sigma), which binds apurinic sites, as evidenced in chemical binding studies (5). Since ARP is a biotinylated molecule that binds to an apurinic site, apurinic sites can be detected using an ELISA reaction with a Streptavidin antibody (GIBCO-BRL) (8). Unfortunately we could not obtain useful results from this assay. In fact, we could not even find predictable results with our positive control, calf thymus DNA which was boiled to create apurinic sites. As a result, this system did not allow us to assay levels of apurinic adducts formed (so that the levels of repaired adducts could be determined following repair by cellular extracts).

Then, studies which utilized 20% denaturing polyacrylamide gels with 8M Urea to identify the presence of apurinic sites or stable adducts in 18 base oligos were attempted. These studies looked at the fragment sizes following E-2,3-Q, E-3,4-Q, or UDG treatment of appropriate radiolabeled oligos. Quantitation of the levels of various sized oligos was then carried out using a PhosphoImager (Molecular Dynamics, CA). The amount of 9-mer (created following E-3,4-Q treatment of the CT9A oligo or the CT9G oligo, or UDG treatment of the GA10dU oligo, resulting in the formation of apurinic sites that break to form a 9mer upon heating) was compared to the amount of 18mer (the length of the original oligo). From these ratios, it was calculated that about 1-2% of the oligos contained apurinic sites. Additionally, similar calculations were carried out for oligos CT9A or CT9G treated with E-2,3-Q, comparing a band slightly higher than the 18mer (which could be the 18mer with a stable adduct attached) and the 18mer. These indicate that, again, about 1-2% of the oligos are reacting with the quinones.

This low level of adduct formation (1-2%) is not surprising, since quinones also react with water. Complicating matters, 1-2% reactions approach the detection limit for gel identification. As a result, a different approach, matrix-assisted laser desorption ionization- time of flight mass spectrometry (MALDI-TOF) was used to better visualize the formation of apurinic sites and stable adducts in DNA by catechol estrogen quinones. These studies are the first, to our knowledge, to directly visualize apurinic sites in DNA, as well as stable adducts (Figure 1). Previously apurinic site formation was surmised by the presence of depurinating adducts or by the binding of reactive reagents, but never directly observed in DNA. These experiments provide the first direct evidence of catechol-estrogen quinone-induced apurinic sites and stable adducts in DNA visualized by MALDI-TOF mass spectrometry.

Once depurination was confirmed with MALDI-TOF mass spectrometry, repair assays were undertaken using GA10dU oligos containing a depurinated dU after UDG treatment. To do these

experiments, GA10dU was complemented with CT9A. This double stranded oligo was then treated with UDG to create an apurinic site in the GA10dU. These samples were then treated with a cell extract from MCF-10 1A cells (using the procedure described by Biade, et al., 9), in the presence of 20uCi radiolabeled TTP, 10U creatine phosphokinase, 250uM dATP, dGTP, and dCTP. A representative gel can be seen in Figure 2. The fact that the repaired oligo is longer than the end-labeled 9mer may be a consequence of nucleotide excision repair, adding bases, but not ligating the oligo closed, or it could be the result of incomplete ligation of the new "repaired" base in its appropriate location. This anomaly is presently being explored by using additional controls.

Further, an *in vivo* repair assay is being created, now that I have successfully demonstrated by MALDI-TOF experiments that catechol estrogens can form stable and depurinating adducts. For the *in vivo* repair assays, an additional template was designed. The 18 base oligos were helpful in the *in vitro* studies, but we decided that a larger template was needed to better explore repair. Although 18 base oligos are long enough for repair proteins in the base excision repair pathway and for the initial proteins of the nucleotide excision pathway to bind, a larger template would also be useful in identifying entire excised regions. As a result, a plasmid containing the green fluorescent protein (GFP) sequence was modified to contain a dU within an essential region for fluorophore activity (10). Integration of the oligo containing the dU was confirmed by sequence analysis. These plasmids cannot be cloned in traditional manners because of the necessary incorporation of dU at a specific location. As a result, they have been prepared directly by preparing multiple batches of plasmid preparations. Plasmids were modified by partial digestion with BsgI to excise a 96 base piece. Then, these cut GFP plasmids, intact GFP plasmids, and complementary 96mer oligos containing a dU are mixed, complemented, and ligated together. The resulting plasmids have also been used as templates to explore *in vitro* repair with cellular extracts. These *in vitro* studies are still underway.

GFP is a protein produced by *Victora Aequophoris*, a jellyfish, and it causes the green glow when the organism is exposed to fluorescent light of 488nm excitation maximum with a 507nm emission maximum. Alterations in the fluorophore region can 1) eliminate activity or 2) change the wavelength of the fluorescing light. The apurinic site generated by dU depurination should eliminate GFP activity. Following microinjection into the MCF-10 A1 cells, plasmids should not have activity, but develop it as the plasmids are repaired over time. In theory, mis-repair will result in a change in fluorescing wavelength. This model provides a wonderful *in vivo* assay of repair systems within living cells. These experiments are just beginning.

In other words, the GFP plasmids containing the dU, which is then depurinated by UDG, is in the process of being utilized for *in vitro* repair assays with cell extracts from MCF-10 1A cells, and for *in vivo* repair following microinjection into MCF-10 1A cells by timing the development of fluorescence.

These studies should shed light on the complex process involved in the identification of damaged bases by DNA repair enzymes. Oligos to be placed in the GFP plasmid are currently being

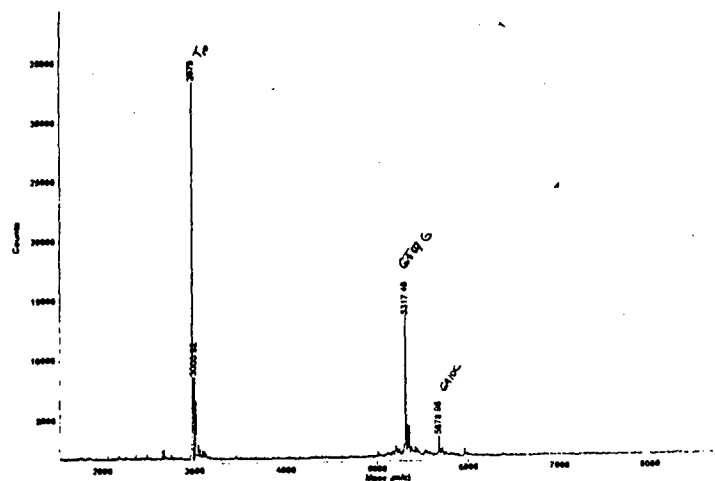
designed to contain a stable adduct at various distances from the apurinic site to determine the impact of having stable adducts and apurinic sites in close proximity in repair assays. Similar 18mers are also being designed. Moreover, now that a system has been devised, determination of the enzymes that are used in this repair can be investigated.

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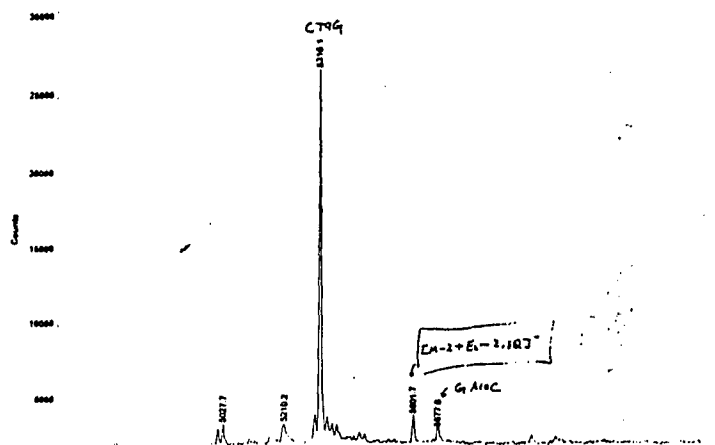
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Figure 1: MALDI-TOF Mass Spectrometry of 18mers

A. Untreated: Untreated CT9G with complementary strand GA10C and size standard T10 (an oligo containing 10 thymines). The peaks correspond to the mass-charge ratios of the intact 18mers of the oligos in this sample.



B. Oligos treated with E-2,3-Q form stable adducts: The labeled peaks represent the starting material CT9G and GA10C. In addition to these peaks, this spectrum also has a peak which is labeled $[M-2+E-2,3Q]^+$ that is representative of a stable adduct forming on the CT9G oligo after treatment with E-2,3-Q.



C. Oligos treated with E-3,4-Q form apurinic sites: This spectrum examines the smaller mass-charge ratios of a E-3,4-Q-treated CT9G oligo. The 8mer, 9mer, and phosphated 9mer (9mer-P) are the appropriate mass-charge ratios to represent the oligo on either side of the apurinic site following its fragmentation by MALDI-TOF mass spectrometry.

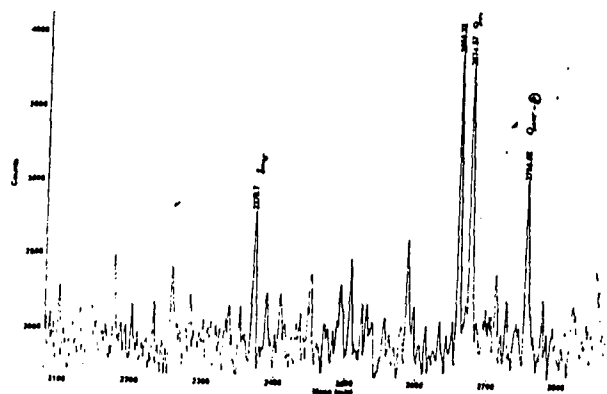
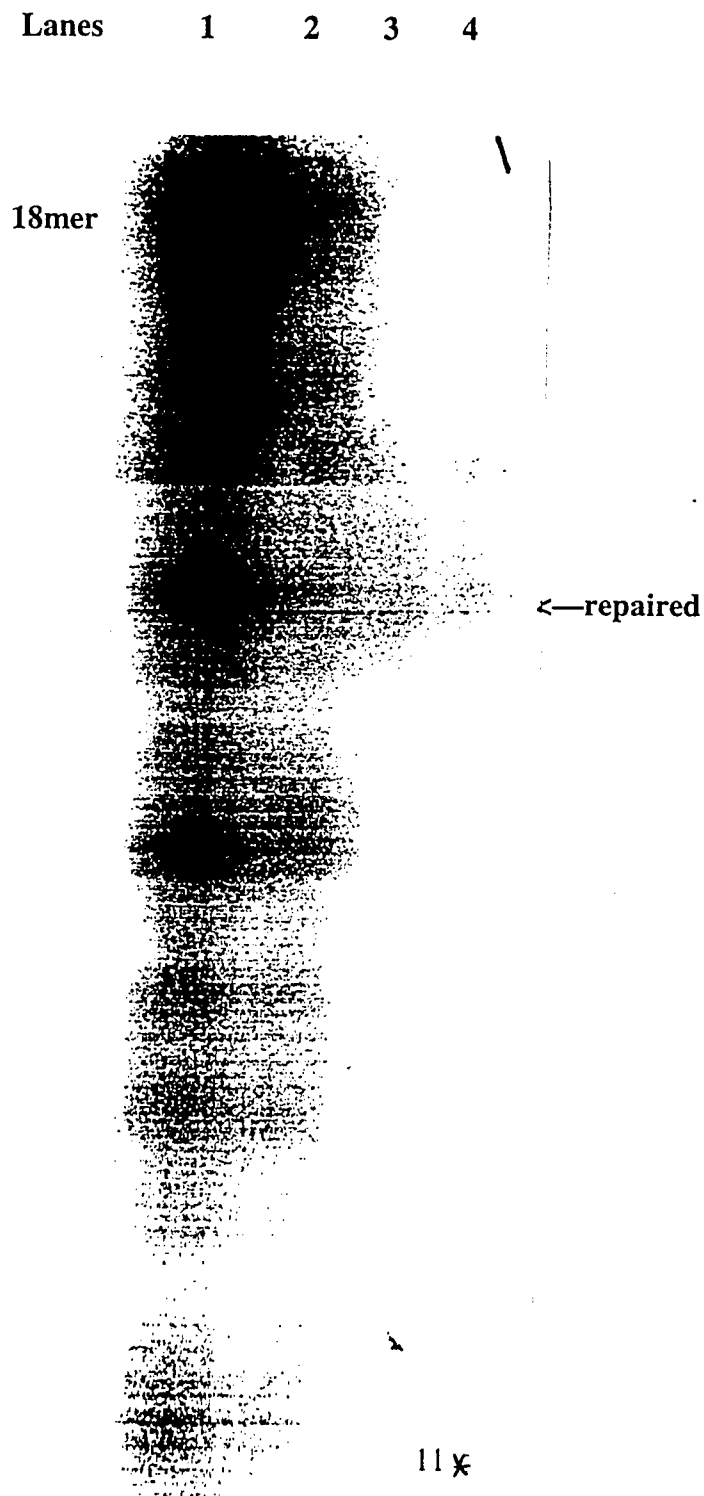


Figure 2: Repair Assay representative gel. Lanes 1 and 2 are untreated [γ - ^{32}P] end-labeled 18mer, GA10dU. Lanes 3 and 4 are GA10dU treated with UDG forming an apurinic site at position 10, treated with cell extract and radiolabeled [α - ^{32}P] TTP.



Appended to the Summary:

Appendix 1: Key Research Accomplishments

Key Research Accomplishments

- Identification of apurinic sites and stable adducts in 18 base long oligonucleotides by MALDI-TOF mass spectrometry
- Identification of repair of apurinic sites in 18 base long oligos by polyacrylamide gel electrophoresis
- Design of an *in vivo* assay system for DNA repair in mammalian cells

Appendix 2. Reportable Outcomes.

Poster/ meeting: (Abstract: Appendix 3a)

Chapman, K. and E. Rogan. "Catechol Estrogen-Quinone-Induced DNA Damage", MD-PhD Student meeting in Aspen CO. July 1998.

Presentation: (Abstract: Appendix 3b)

Chapman, K. "Catechol Estrogen DNA Damage", Eppley Institute for Research in Cancer Student/Post-doc Seminar Series, October 1998.

Paper: (Abstract: Appendix 3c)

Chapman, K., L. Zhang, M. Gross, E.C. Cavalieri, and E. Rogan. Identification of estrogen quinone-induced apurinic sites and stable DNA adducts by MALDI-TOF mass spectrometry. In preparation

Appendix 3a:

MD-PhD student meeting, Aspen, CO July 1998.

Poster title: Catechol Estrogen DNA Damage

Authors: **Kimberly Chapman** and Eleanor Rogan

ABSTRACT

Evidence from our laboratory and others indicates that estrogens act as complete endogenous carcinogens in initiating and promoting the development of cancer. Estradiol or estrone can be oxidized to its catechol form (CE) at the 2 or 4 position by cytochrome P450s and then oxidized by cytochrome P450s or peroxidases to quinone forms (CE-Q) (See figure 1).

These CE-Q damage DNA by directly binding to the DNA and forming stable adducts which remain in the DNA or depurinating adducts which are lost when the bond between the sugar and nucleobase is broken (See figure 2). *In vitro* and *in vivo* assays indicate that these CE-Q bind to the N7 of guanine or the N3 of adenine to form depurinating adducts that lead to apurinic sites in the DNA. CE-Q-initiated stable adducts form at the 2-amino of guanine and the 6-amino of adenine. CE-2,3-Q form only stable adducts, whereas CE-3,4-Q form more than 99% depurinating adducts.

DNA damage by CE-Q in ACI rat mammary glands treated in culture with CE-2,3-Q and CE-3,4-Q was demonstrated by [³H]thymidine incorporation. Treated glands demonstrated higher levels of incorporation than untreated glands. Studies were then undertaken to develop molecular biological methods to visualize CE-Q-induced DNA damage. Using 18-base single-strand oligonucleotides, including one with a uracil-DNA glycosylase-induced apurinic site, we showed that electrophoresis on 20% polyacrylamide denaturing gels can be used to visualize apurinic site formation in these oligos. This method is being used to visualize CE-Q-induced apurinic site formation. Once the CE-Q-induced damage is visualized, double strand oligos containing apurinic sites will be treated with cellular extracts from MCF-10A1 cells to see whether DNA repair can be initiated and visualized using this gel electrophoresis technique.

Appendix 3b:

Abstract for seminar presented by **Kimberly Chapman** in October 1998 at the Eppley Institute Student/post-doc seminar series

Endogenous carcinogens, cancer-causing agents formed in mammalian cells, have been poorly investigated. Our group has hypothesized and furnished evidence that oxidized metabolites of catechol estrogens (CE) initiate breast cancer by damaging DNA in breast cells. These active metabolites, the quinones of CE (CE-Q), bind to DNA and form two types of adducts: depurinating adducts that generate apurinic sites, and stable adducts that remain linked to DNA. It is logical to speculate that for the progression from DNA damage to mutation, the repair

mechanisms within cells must sometimes fail. The main objective of these experiments is to demonstrate CE-Q-induced DNA damage and to shed light on the mechanism of faulty repair. First I have determined the level of DNA damage by CE and the cell type which underwent this damage by use of an unscheduled DNA synthesis assay in ACI rat mammary gland organ culture. Then, I have tried to design molecular biological techniques that display DNA damage. The most successful technique uses a series of radiolabeled single-strand oligonucleotides which contain a single site that can undergo CE-induced DNA damage creating strand breaks that are visualized on a polyacrylamide gel.

Appendix 3c:

Paper in Preparation

Title: Identification of estrogen quinone-induced apurinic sites and stable DNA adducts by MALDI-TOF mass spectrometry

Authors: **Kimberly A. Chapman**, LiKang Zhang, Michael L.Gross, Ercole L.Cavalieri, and Eleanor G. Rogan

Abstract:

This article reports for the first time apurinic sites and stable adducts directly identified in DNA by means of matrix assisted linear desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The estrogen metabolite estradiol-3,4-quinone (E_2 -3,4-Q) was used to create depurinating adducts that generate apurinic sites and estradiol-2,3-quinone (E_2 -2,3-Q) was used to create the stable adducts. Spectra taken after the E_2 -3,4-Q reacted with an 18 base oligonucleotide containing a single adenine or guanine indicate the formation of a predictable apurinic site that in MALDI-TOF mass spectrometry is converted into a strand break. In contrast, results with the same oligodeoxynucleotides reacted with E_2 -2,3-Q indicate the formation of a stable adduct. These results suggest that metabolites of endogenous estrogens can react with DNA to cause damage that may lead to mutations.



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